Article

Development of Antibacterial, Antioxidant, and UV-Barrier Chitosan Film Incorporated with *Piper betle* Linn Oil as Active Biodegradable Packaging Material

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Citation: Nguyen, T.T.; Nguyen, T.-T.T.; Tran, T.V.; Tan, L.V.; Danh, L.T.; Than, V.T. Development of Antibacterial, Antioxidant, and UV-Barrier Chitosan Film Incorporated with *Piper betle* Linn Oil as Active Biodegradable Packaging Material. *Coatings* **2021**, *11*, 351. <https://doi.org/10.3390/coatings11030351>

Academic Editor: Maria Jose Fabra

Received: 14 January 2021

Accepted: 5 February 2021

Published: 19 March 2021

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Abstract: This study aims to introduce the antibacterial and antioxidant activities of the *Piper betle* Linn oil (PBLO) into chitosan film (pCS), named as pCS-PBLO film. The morphology, structure, and properties of the pCS-PBLO film, along with the PBLO concentration between 0.4% and 1.2% (*v/v*), were determined. The film surface became rough and heterogeneous with the addition of PBLO, which directly influenced on mechanical strength of the resultant film. The addition of the PBLO did not affect thermal stability but significant effect on flexibility and mobility of the film. Importantly, the film enhanced the UV-protective property and antioxidant activity as incorporated-PBLO. Moreover, the resulting film revealed the great inhibition efficiency against the negative-gram (*E. coli*, *P. aeruginosa*, and *S. typhi*) and positive-gram (*S. aureus*) bacteria based on phenolic compounds, such as the acetyleugenol, eugenol, 4-allyl-1,2-diacetoxybenzen, and chavicol acetate in PBLO components. In particular, the pCS-PBLO film may extend the shelf life of king oranges up to two weeks at 25 °C that is longer as compared to the uncoated sample and coated with chitosan alone. These results suggest that the pCS-PBLO film can be used as environmental-friendly and effective food packaging material in the future.

Keywords: antibacterial activity; antioxidant activity; chitosan film; *Piper betle* Linn oil; UV-barrier property

Highlights

Successful development of chitosan film incorporated with *piper betel* Linn oil as active food packaging material

The pCS-PBLO film possessed thermal stability and stretchability

Impressive UV-barrier function was found in the pCS-PBLO film

The pCS-PBLO film showed antibacterial and antioxidant activities

1. Introduction

Petroleum-based plastics are one of the main contributors to serious environmental pollution due to their un-degradable characters. It was found that thousands of marine

wildlife were yearly killed by ingesting plastic-based pollutants or getting entangled [1]. To address these problems, the recent development of biodegradable and environmental-friendly materials as interesting alternatives to conventional plastics has been paid much attention in food packaging area. Biodegradable plastics were abundantly developed from polysaccharides such as chitosan, chitin, starch, cellulose, pectin [2]. In response to increasing people's standard of living and productivity growth in agricultural production, active packaging produced by the incorporation of antibacterial and antioxidant agents has become equally crucial interests, when food-borne pathogen is the major cause of food deterioration limiting the supply of nutritious and security foods [3,4]. The combination of natural materials to produce environmental-friendly and active coating to prolonging the shelf life of food, has been developed and considered as a favorable technology in food preservation [5,6].

Piper betle Linn, a traditional herb from *Piperaceae* family, is widely found in India, Indonesia, Malaysia, Philippines, Sri Lanka, and other Southeast Asian and East African countries [7]. The major components of *Piper betle* Linn is complex mixture of polyphenols, flavonoids, tannins, and polysaccharides [8]. Phytochemical compounds such as hydroxy chavicol, 4-chromanol, and eugenol were found consistence in India betle leaves which offered great antioxidant, anti-inflammatory, antibacterial, and antifungal properties [9]. The betle essential oil has been also reported to be the potential sources to produce ayurvedic medicine and herbal products due to containing chavibetol, caryophyllene, and methyl eugenol components [10]. Furthermore, *Piper betle* Linn oil was also known as food additive for the preservation and extension of the shelf life of dry fruits, spices, and areca nut [11].

Chitosan is a product from the deacetylation of chitin which is abundantly obtained from the natural sources such as the shells of crustaceans, arthropod, and fungi [12]. Chitosan possesses antibacterial, antifungal, biodegradable, and biocompatible characters because it's structure composed of β -1,4-linked glucosamine and N-acetylglucosamine [13]. In general, chitosan contains the charged groups which can interact with the peptidoglycans in the microorganism wall causing the leakage of intracellular electrolytes and cell death [14,15]. Interestingly, chitosan is recognized as food additive as listed in Generally Recognized as Safe (GRAS) by US food and Drug Administration in 2013 and thus it is ideally suitable for packaging material in directly contacting with foods. Furthermore, the excellent film-forming property of chitosan makes it to become ideal candidate for active packaging area. Recently, much effort on the introduction of essential oils into chitosan film to produce packaging film possessing antibacterial and antioxidant function has been reported in different studies. The addition the chitosan film with the cinnamon oil (CO) [at concentration of 0.4–2% (v/v)] displayed antibacterial activity and moisture barrier property, in which the low flexibility and mobility of blend film with further increasing CO content was a negative mark [16]; the *Zataria multiflora* Boiss oil showed antioxidant activity and water barrier property, however the film displayed poor mechanical behavior [17]; the basil essential oil [at concentration of 0.1–0.5% (v/v)] enhanced antibacterial activity and water vapor barrier property of resultant film but decreased in mechanical property of the film [18]; the *Perilla frutescens* (L.) Britt. essential oil [at concentration of 0.2–1% (v/v)] imparted water barrier and antibacterial function but also revealed a decrease in mechanical property of the film [19]; the *Eucalyptus globulus* essential oil [at concentration of 1–4% (v/v)] endowed both antibacterial and antioxidant activities [20]; and in a more recent work, the anise (*Pimpinella anisum* L.) essential oil [at concentration of 0.5–2% (v/v)] enhanced mechanical property, water barrier property, and antibacterial activity [21]; and the Apricot (*Prunus armeniaca*) kernel essential oil [at concentration of 0.25–2% (v/v)] enhanced water barrier, antibacterial, antioxidant, and antifungal property [22].

In our previous finding, the *Piper betel* Linn leaf extract (PBLLE) was successfully introduced into chitosan film uninvolved plasticizer to impart outstanding antibacterial activity, antioxidant activity, and good resistance to water vapor permeation to prepared films [23]. Herein, we found that the utilization of *piper betel* Linn oil (PBLO) to chitosan film with glycerol-based plasticizer induced different behaviors in bioactivity and physical

property of resultant film as compared to the addition of PBLLE because of high hydrophobicity of PBLO. Interestingly, the light of present work was high transparent of film made from chitosan and PBLO in visible region as compared to chitosan film containing PBLLE in dark brownish which significantly affected to see-through property important for food packaging material as customer want to see packaged product before buying. Besides to this, the combination of nontoxic components including chitosan and *Piper betel* Linn oil is considered to be safe material for food packaging.

2. Experimental

2.1. Materials

Chitosan (DD 75–85%, and $M_w \sim 50$ –190 kDa), glycerol ($\geq 99.5\%$), Folin-Ciocalteu's phenol reagent ($\geq 98\%$), sodium carbonate ($\geq 99\%$), sodium chloride ($\geq 99\%$), 2,2-diphenyl-1-picrylhydrazyl (97%), and gallic acid ($\geq 98\%$) were supplied by Sigma-Aldrich (Missouri, MO, USA). Ethanol (99.7%), Tween 80 (98%) and methanol (99.7%) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). *Piper betel* Linn leaf was collected from Hau Giang province, Vietnam and extracted oil through hydrodistillation, followed by the store at -2°C until use. Bacterial strains including *Staphylococcus aureus* (*S. aureus*) NRRL B-313, *Pseudomonas aeruginosa* (*P. aeruginosa*) NRRL B-14781, *Escherichia coli* (*E. coli*) NRRL B-409, and *Salmonella typhimurium* (*S. typhimurium*) YS1646 were provided by Institute of Tropical Biology, Vietnam Academy of Science and Technology (Ho Chi Minh, Vietnam).

2.2. Analyses of PBLO

Chemical composition of PBLO was determined by using GC Agilent 6890N instrument (California, CA, USA) equipped with MS 5973 inert and HP5-MS column. The head column pressure was 9.3 psi and helium worked as the carrier gas with velocity of 1 mL/min. Before testing, 25 μL of PBLO was mixed in 1 mL of n-hexane and further dehydrated by Na_2SO_4 . The injection temperature was set at 250°C and split injection in the ratio of 1:100. Thermal profiles were set up as follows: the initial column temperature at 50°C for 2 min, continued up to 80°C at velocity of $2^\circ\text{C}/\text{min}$, continually increasing to 150°C at velocity of $5^\circ\text{C}/\text{min}$, and followed to increase to 300°C at velocity of $20^\circ\text{C}/\text{min}$ for 5 min. The individual compound was identified and confirmed by comparing to those of available library data from GC/MS system.

2.3. Preparation of pCS-PBLO films

Chitosan solution was produced by dissolving 1 g of chitosan powder in 100 mL of aqueous acetic acid solution (1%, v/v). The solution was magnetically stirred at 800 rpm and room temperature (at around 27°C) for 24 h to dissolve completely solids. Glycerol (30%, w/w) was then added into chitosan solution with the function of plasticizer. Subsequently, the PBLO was added into plasticized chitosan solution at different volume ratio [0.4%, 1%, and 1.2% (v/v)] before adding 0.2% (w/v) of Tween 80 as an emulsifier. Finally, film-forming solution was casted on the $20\text{ cm} \times 20\text{ cm}$ plastic mold and further dried at 40°C for 48 h. After drying, the chitosan-based blend films were peeled off and kept at 25°C for further analyses. The control chitosan film was denoted as pCS and chitosan films containing PBLO were remarked as pCS-0.4PBLO, pCS-1PBLO, and pCS-1.2PBLO with respect to incorporated PBLO content at 0.4%, 1%, and 1.2% (v/v). The thickness of films was controlled by taking equal volume in film fabrication process to ensure ignorable variation and thickness values were recorded at 0.053 ± 0.002 ; 0.055 ± 0.003 ; 0.064 ± 0.003 ; and 0.078 ± 0.004 , respectively for pCS, pCS-0.4PBLO, pCS-1PBLO, and pCS-1.2PBLO.

2.4. Film Characterization

The surface morphology of the as-prepared films was recorded using scanning electron microscope (SEM) on JSM-IT200 equipment (Massachusetts, MA, USA). The attenuated total reflection (ATR)/Fourier transform infrared (FTIR) (ATR/FTIR) spectra of

the as-prepared films were determined using a JASCO FT/IR-6600 instrument (JASCO International Co., Tokyo, Japan) in the wavenumber ranged from 4000–500 cm^{-1} . The thermo-stability of the as-prepared films was determined using the thermogravimetric analyses (TGA) and derivative thermogravimetric (DTG) on a TGA model Q500 V20.10 Build 36 (TA Instruments, Hüllhorst, Germany) by a temperature program of 27–800 $^{\circ}\text{C}$ with heating rate of 10 $^{\circ}\text{C}/\text{min}$. The crystal structure of the as-prepared films was determined using X-ray diffraction (XRD) on a Bruker D8 Advance powder diffractometer (Bruker AXS GmbH, Berlin, Germany) with $\text{Cu-K}\alpha$ radiation source and 2θ range of 2–60 $^{\circ}$ with 0.2 s/step. The mechanical behavior of the as-prepared films was measure on AG-Xplus Series Precision Universal Testers following to ASTM-D882 standard [24] with the size film of 15 mm $\times$ 100 mm and gauge length of 50 mm and crosshead speed of 10 mm/min at room temperature (at around 27 $^{\circ}\text{C}$). The transmittance of the as-prepared films was recorded on UV-1800 UV/Visible Scanning Spectrophotometer (Shimadzu Scientific Instruments Inc., Kyoto, Japan) in wavelength range of 190–800 nm.

Swelling degree (SD) was performed based on the method previously described [25]. Five pieces (2 cm $\times$ 2 cm) of film were initially dried at 70 $^{\circ}\text{C}$ for 24 h and weighted to get dry weight denoted as m_1 . Then, the dried pieces of film were soaked in 30 mL of distilled water for 24 h at room temperature (27 $^{\circ}\text{C}$). For next step, the pieces of film were separated from water and removed the residual surface water using tissue paper and further weighted (m_2). The SD (%) was calculated by following equations:

$$\text{SD (\%)} = \frac{m_2 - m_1}{m_1} \times 100\% \quad (1)$$

2.5. Bacterial Inhibition Test

The antibacterial activities of the pCS and pCS-PBLO films were tested using the agar diffusion and liquid incubation methods as previously descriptions [25,26]. For the agar diffusion tests, a 100 μL (an approximately titer of 2×10^6 colony forming units per mL, CFU/mL) of each bacterial strain, including the *S. aureus*, *S. typhimurium*, *E. coli*, and *P. aeruginosa*, was spread onto the LB agar plates (Sigma-Aldrich, Missouri, MO, USA) in triplicate. A 1-cm² slide of each pCS and pCS-PBLO films were then added onto midpoint of the plates (Supplementary Figure S1). Subsequently, the plates were incubated at 37 $^{\circ}\text{C}$ for 24 h. The inhibition zone diameters were measured with a ruler.

For the liquid incubation tests, only the *E. coli* strain was used. A 2-cm² slide of each pCS and pCS-PBLO films was added into the tubes containing 2 mL of the LB medium. The 5% (v/v) of bacterial suspension at density of 2×10^8 CFU/mL was added into the tubes. The tubes were incubated in sharking incubator at 37 $^{\circ}\text{C}$ and 200 rpm. The medium was collected at different incubated times of 0, 4, 8, 11, and 24 h, and direct spread onto the agar plates in triplicate to evaluate the bacterial viability.

$$A\left(\frac{\text{CFU}}{\text{mL}}\right) = \frac{N}{3} \times f \quad (2)$$

(where: A, total colonies in 1 mL of sample; N, total colonies in three agar plates; and f , the dilution factor).

2.6. Antioxidant Test

The antioxidant activity of chitosan solution with and without PBLO was determined using 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH $\cdot$) method previously described by Brand-Williams et al. [27]. Initially, the chitosan-PBLO solutions were diluted in distilled water to obtain the different concentration seri (500–32.25 $\mu\text{g}/\text{mL}$). The diluted sample solution (1 mL) was added into 1 mL of 0.3 mM DPPH $\cdot$ methanol solution and further well-vortexed for 7 s before dark-incubating for 60 min at ambient temperature (27 $^{\circ}\text{C}$).

After dark-incubated, the UV-Vis absorbance of mixture solution was measured at 517 nm. The scavenge efficiency to DPPH free radical was calculated in the following equation:

$$\text{Scavenge efficiency (SE\%)} = \frac{\text{OD}_c - \text{OD}_t}{\text{OD}_c} \times 100 \quad (3)$$

(where OD_c and OD_t are respectively the absorbance of 0.3 mM DPPH· methanol solution and the chitosan-PBLO solution).

Antioxidant activity of PBLO containing chitosan film was evaluated through IC_{50} ($\mu\text{g/mL}$) which was determined from the plot of working sample concentration versus SE%. The experiments were replicated in triplicates to get an average IC_{50} parameter for each composition.

2.7. Surface Tension, Work of Adhesion, and Spreading Coefficient

The coating efficiency of the coating solution was evaluated by the work of adhesion and spreading coefficient due to contact angle measurement of coating liquids over orange skin [28]. Surface tension of coating solution and contact angle of all chitosan solution with and without PBLO over orange skin was determined by using a Theta Optical Goniometer (Jersey, NJ, USA). The adhesion and spreading coefficient were calculated according to the Wenzel equation [29] as follows:

$$\text{Work of adhesion} = \gamma_1(1 + \cos \theta) \quad (4)$$

$$\text{Spreading coefficient} = \gamma_1(\cos \theta - 1) \quad (5)$$

where γ_1 and θ are respectively the surface tension of coating solution and the contact angle of the film coating on the orange skin.

2.8. Coating of King Oranges

King oranges were pre-treated by washing with distilled water and further blow-dried under a fan at ambient temperature (27 °C). The king oranges were coated by chitosan and chitosan-PBLO solution using the pressure-spraying method. The uncoated oranges were used as control sample. All coated samples were then stored at 25 °C and daily evaluated the appearance changes for 2 weeks.

3. Results and Discussion

3.1. Chemical Composition of Piper betle Linn oil

The volatile profile of *Piper betle* Linn oil (PBLO) was determined using GC-MS (Table 1). Thirty-eight various compounds were composed 98% of total oil. It can be seen that the phenolic components abundantly accounted for highest area percentage (48%) of total oil, including acetyleugenol (17.51%), eugenol (14.29%), 4-allyl-1,2-diacetoxybenzen (10.92%), chavicol acetate (3.00%), 3-allyl-6-methoxyphenol (1.82%), and 4-allylphenyl acetate (0.42%). These results indicated that the eugenol and its derivative were main components in PBLO which were possibly used as natural antibacterial and antioxidant agents in food packaging area. Similar finding was found in *Piper betle* var. *magahi* oil collected from Varanasi, India reported by Prakash et al. [11] mainly consisted of phenolic compounds such as eugenol (63.39%), acetyleugenol (14.05%), 4-allyl-1,2-diacetoxybenzen (0.13%), and chavicol (0.55%) meanwhile this variety only contained eugenol 44.14% and chavicol 1.52% when it was harvested from West Bengal, India [30]. The differences in phenolic content and types came from the ecological and geographical regions, age of leaves, harvesting season, drying, and extraction conditions [31].

Table 1. Chemical composition of *Piper betle* Linn. leaf oil.

No.	Retention Time (min)	Compound Name	Area Percent (%)
1	7.261	α -Pinene	1.051
2	7.837	Camphene	0.314
3	9.081	β -Pinene	0.171
4	11.862	Eucalyptol	1.376
5	16.15	β -Linalool	0.36
6	26.481	δ -Elemene	0.364
7	26.847	Chavicol acetate	3.004
8	27.088	4-Allylphenyl acetate	0.422
9	27.642	Eugenol	14.209
10	27.673	Copaene	3.098
11	27.746	3-Allyl-6-methoxyphenol	1.824
12	27.977	Geraniol acetate	1.19
13	28.227	β -Elemene	2.343
14	28.928	α -Bergamotene	0.952
15	29.043	Caryophyllene	9.107
16	29.315	β -copaene	0.486
17	29.514	α -Bergamotene	0.711
18	29.597	Aromadendrene	0.794
19	30.016	α -Caryophyllene	1.732
20	30.152	β -Farnesene	0.383
21	30.695	γ -Murolene	6.015
22	30.81	Germacrene D	3.945
23	30.904	2-Isopropenyl-4a,8-dimethyl 1,2,3,4,4a,5,6,7-octahydronaphthalene	0.998
24	31.208	β -Cyclogermacrene	3.964
25	31.333	α -Murolene	1.053
26	31.542	β -Bisabolene	1.34
27	31.678	γ -Cadinene	0.767
28	31.908	β -Cadinene	3.361
29	32.086	Acetyleugenol	17.511
30	32.327	α -Calacorene	0.777
31	32.902	Palustrol	0.24
32	33.247	Globulol	0.589
33	33.404	Viridiflorol	0.446
34	33.602	cis-Eudesm-6-en-11-ol	0.228
35	33.916	Junenol	0.151
36	34.073	1,10-Diepicubenol	0.33
37	34.366	4-Allyl-1,2-diacetoxybenzene	10.922
38	34.543	α -Cadinol	0.948

3.2. ATR/FTIR Analyses

ATR/FTIR spectroscopy was used for the purpose of identifying chemical functional groups and determining possible interaction between components in polymeric blend. The spectrum of film made from chitosan alone presents an absorption band in the range of 3624–3012 cm^{-1} due to the overlapping of O–H and N–H stretching vibration (Figure 1) [32]. The C–H antisymmetric and symmetric stretching vibrations of $-\text{CH}_2$ groups were recorded at 2873 cm^{-1} [33]. The characteristic chitosan signs were observed at 1650 cm^{-1} belonging to C=O stretching vibration in amide-I, 1532 cm^{-1} corresponding to C–N stretching vibration composed in amide-II and N–H bending vibration, and 1400 cm^{-1} (HN–CO stretching vibration in amide-III) [32,34]. Meanwhile the sharp peak at 1020 cm^{-1} was related to C–O stretching vibration [34]. It was found that appreciable changes in chitosan ATR-FTIR spectrum by the addition of PBLO. For instance, the absorption band at 3624–3012 cm^{-1} (O–H and N–H stretching vibration) became weaker and more flatted by the addition of PBLO. The decrease in the peak intensity at 1533 cm^{-1} corresponding to C–N stretching vibration and 1402 cm^{-1} (NH–CO stretching vibration) by the addition of PBLO along with the significant evolution of shoulder at 1584 cm^{-1} due to the aromatic

ring vibration of phenolic compounds [33]. Furthermore, the absorption band at 1020 cm^{-1} (corresponding to C–O stretching vibration) in the film formulation made from chitosan and PBLO shifted to higher frequency region and became more intense and compared to those in control chitosan spectrum when PBLO abundantly contained C–O groups in structure of phenolic compounds. These changes were likely ascribed to the hydrogen-bonding interaction between O–H and N–H groups in chitosan and hydroxyl of phenolic compound in PBLO [33]. Murat Kaya et al. [33] also found the possible hydrogen interactions arising between film functional groups and *Berberis crataegina's* seed oil evidenced by the slight shift in the C–O stretching vibration peak to a higher frequency region when seed oil was incorporated into chitosan.

Figure 1. The ATR/FTIR analyses of (a) pCS, (b) pCS-0.4PBLO, (c) pCS-1PBLO, and (d) pCS-1.2PBLO.

The ATR/FTIR results indicated the existence of inter-molecular interaction between functional groups in PBLO and chitosan, which could be expected to diminish oil release into environment and thus allowing maintaining antibacterial and antioxidant activity of blend films during food protection.

3.3. XRD Analyses

Possible structural changes in chitosan network with the addition of PBLO were confirmed by XRD (Figure 2). The film made from chitosan alone reveals characteristic peaks at 11.4° and 18.1° , which are closely related to anhydrous and hydrated crystalline [7,35], and a typical fingerprint at 20.8° attributed to amorphous state of chitosan [36,37]. The disappearance of crystal peaks at 11.4° and 18.1° and the increase in diffraction intensity at 20.8° as the addition of 0.4% (v/v) and 1.2% (v/v) PBLO imply a decrease in crystallinity of blend film. It is likely suggested that inter-molecular interaction between phenolic groups in PBLO and chitosan functional groups hinder and disturb intra-molecular interaction between chitosan chains resulting in the predominance of amorphous region [34]. Besides to this, the new pronounced diffraction peak was identified at 14.4° with 1.2% (v/v) PBLO addition, probably indicating that such inter-molecular interactions exert the rearrangement in polymeric network to generate newly ordered structure. The similar behavior in the reduction in crystalline was documented in chitosan consisted of polyphenols from thinned young apple [37]. However, re-evolution of the crystalline peaks at 11.4° and 18.1° with higher diffraction intensity in chitosan film containing 1% (v/v) PBLO is still unclear, it is likely to suggest that the position of original ordered chains in control chitosan are replaced by a large amount of crosslinking network between chitosan and PBLO. It can be

therefore concluded that the addition of PBLO may convert semi-crystalline in chitosan film toward more amorphous structure, which significant affect to mechanical behavior of the blend film in further investigation.

Figure 2. XRD analyses of pCS, pCS-0.4PBLO, pCS-1PBLO, and pCS-1.2PBLO.

3.4. Thermo-Stability

TGA and DTG analyses were utilized for the purpose of investing the thermal stability of film made from chitosan alone and containing PBLO. As seen in TGA curves (Figure 3a), the control chitosan film presents three-stage decomposition. The first stage at 50–150 °C was due to the vaporization of adsorbed water on the film surface (50–80 °C) and residual acetic acid (80–150 °C) [19,38]. The decomposition of glycerol-based plasticizer occurred at 150–250 °C [19]. The main degradation stage at 250–360 °C with 43% weight loss (Table S1) was attributed to the saccharide ring dehydration and decomposition of chitosan backbone [19]. In case of chitosan with PBLO, there are three steps of decomposition were documented at 50–150 °C, 150–360 °C, and 360–450 °C. It should be noticed that the decomposition of glycerol is negligible in the TGA curves of blend films and the newly decomposition stage not found in chitosan alone appears at 400–450 °C due to the decomposition of aromatic compounds in PBLO and surfactants [34,38,39]. The difference in thermal behavior of chitosan film with and without PBLO was pronouncedly observed from DTG profile. In details, the decomposition peak of chitosan backbone was recorded at 296 °C and 284 °C for control chitosan and pCS-PBLO film, respectively. This result indicated that the thermal stability of chitosan film slightly decreased by the addition of PBLO. More interesting, it was not found the PBLO own degradation peak. This was likely pointed that PBLO was well-embedded in blend film due to the inter-molecular interaction between phenolic compounds and chitosan functional groups resulting in good protective effect and preventing separate decomposition of PBLO.

Figure 3. (a) TGA and (b) DTG profiles of pCS, pCS-0.4PBLO, pCS-1PBLO, and pCS-1.2PBLO.

3.5. The Surface Morphology

The surface images at micro level of film formulation made from chitosan alone and containing 0.4–1.2 % (v/v) PBLO are observed in Figure 4. Herein, glycerol was additionally used as plasticizer for all the films and the successful film formation for all applied formulations was obtained as expected. The control chitosan film without PBLO displayed uniform, smooth, homogeneous appearance without cracks and fractures, indicating compact and continuous structure. Meanwhile the film made from blend of chitosan and PBLO presented the rough and heterogeneous surface with the appearance of spherical-like droplets, resulting in discontinuities in blend film that may affect to weak fracture resistance of film. The existence of spherical-like droplets indicated the dispersion of lipid droplets on the surface of resulting films. However, the spherical-like droplets

were not found on the surface of chitosan film incorporated with *Piper betle* Linn leaf extract [23,40]. Similar findings were found in chitosan film containing 0.4–1.2% (*w/v*) *Origanum vulgare* L. oil [30], and 1–4% (*v/v*) *Eucalyptus globulus* oil [20], when spherical oil droplets tended to separate and center at the upper surface of film, likely resulted from the traction force caused by chitosan matrix during drying step. At lower PBLO content (0.4%, *v/v*), lipid droplets were scarcely observable since they abundantly concentrated on the surface of film with small droplet size. As increasing PBLO concentration to 1% (*v/v*), droplet size slightly enlarged and became visible. However, the film surface became more heterogeneous when some spherical-like droplets were embedded and trapped in the polymeric network. The slight increase in droplet size may due to the fact that flocculation and coalescence occurred at higher lipid content [41]. Although droplet size of lipids was lower, the surface of chitosan film containing 1.2% (*v/v*) PBLO become more homogeneous as compared to that in inclusion of 0.4% (*v/v*) and 1% (*v/v*) PBLO. The decrease in lipid size likely due to well-embedded oil droplets in polysaccharide matrix resulting in the change in the shape of lipid droplets. This observation was coincident with what was seen gelatin–chitosan containing *Origanum vulgare* L. essential oil (OEO) as higher droplet size was found in film incorporated with 0.8%(*w/v*) OEO than that in 0.4 (*w/v*) and 1.2 (*w/v*) incorporated-OEO [42].

Figure 4. SEM images of (a) pCS, (b) pCS-0.4PBLO, (c) pCS-1PBLO, and (d) pCS-1.2PBLO.

3.6. UV-Protective Property

The high transparent and see-through property are important for food packaging material because it easily attract customer who want to see packaged product before buying. The UV barrier property is of great benefit to protect food preventing photo-oxidation and degradation of vitamin and other photosensitizers [43]. The light transmittance in UV-Vis of 200–800 nm for chitosan-based films is displayed in Figure 5. The film made from chitosan alone presents low transmittance in the UVC region (250–280 nm) on the ground of the existence of double bond (C=O) in remaining chitin after the deacetylation [44]. It was also found that about 90% light in visible region (400–800 nm) transmitted though control chitosan film meanwhile the chitosan incorporated with 0.4–1.2% (*v/v*) PBLO presented the light transmittance of 70% in this band. Interestingly, the light transmittance of film formulation made from chitosan containing 0.4% (*v/v*) and 1% (*v/v*) PBLO was near zero in the region of UVC (250–280 nm) and UVB (280–320 nm) and enlarged to UVA (320–400 nm) with 1.2% (*v/v*) incorporated-PBLO. This is due to UV absorbance of phenolic compounds bearing hydroxyl groups conjugated aromatic rings. Similar behavior was also documented as combining chitosan and turmeric extract (mainly composed curcuminoids) which acted as UV absorber due to aromatic groups in the curcuminoids [6]. It can be therefore concluded that film made from chitosan and PBLO can completely block the UV light without significantly reducing the see-through character of food packaging material, which was improved as compared to chitosan film incorporated with *Piper betle* Linn leaf extract in our previous report [23].

Figure 5. UV-Vis analyses of the pCS, pCS-0.4PBLO, pCS-1PBLO, and pCS-1.2PBLO.

3.7. Mechanical Behavior

Sufficient mechanical property of edible films makes an important contribution to protect the integrity of packaging materials during storage and transportation. The tensile strength (Ts), elongation at break (Eb), and elastic modulus (Em) of chitosan-based blend films were recorded at 15.26 MPa, 17.49%, and 111.96 MPa, respectively (Table 1). The incorporated-PBLO caused a considerable decrease in Ts (15.26–5.42 MPa) and Em (111.96–25.95 MPa). This behavior is likely due to the presence of oil droplets as observed in SEM images, hindering and interfering chain-chain interaction in chitosan resulting in discontinuities in polymeric chains and thus allowing decreasing fracture resistance and stiff of blend film. Furthermore, the weak inter-molecular interaction between chitosan and PBLO which may partially alter powerful chain-chain interactions [45]. Notably, the film made from chitosan and PBLO became more flexible and mobile evidenced by increasing elongation at break (17–25%) compared to the film without PBLO. This behavior was partially due to the presences of lipid droplets in film matrix which hinder polymer chain-chain interaction and result in flexible domains in film [46]. Furthermore, phenolic components interacted with chitosan functional groups and weaken the intra molecular forces among polymeric chains resulting in the increment in mobility and flexibility of chitosan film [45]. Another possible explanation due to the presence of emulsifier like Tween 80, importantly contributed to plasticizing effect as synergistic effect between glycerol surfactants with small molecules which could stay between polymeric chains and increase the chain mobility [47]. It was found that the film containing chitosan and PBLO offered more flexibility and mobility in comparison with chitosan/*Piper betle* Linn extract [23]. Similar findings were reported based on the addition of thyme and clove essential oil into chitosan film [48] and *Origanum vulgare* L. essential oil into gelatin-chitosan [42] that induced a reduction in Ts although a significant increase in Eb was observed. In contrast, the film made from chitosan incorporated cinnamon essential oil [16], and Apricot (*Prunus armeniaca*) kernel essential oil [22] presented an increase in Ts and decrease in Eb compared to chitosan alone. Whereas other essential oils such as *Perilla frutescens* (L.) Britt. essential oil [19], basil essential oil [18], *Zataria multiflora* Boiss essential oil [17] caused a significant reduction in both Ts and Eb. These differences as essential oils were added into simple chitosan film [17–19] on mechanical behavior may be mainly due to different kinds and components contained in essential oil, testing conditions as well as emulsifying agents [19].

In general, the addition of PBLO into chitosan resulted in more flexible and mobile blend films as merit for food packaging material.

3.8. Swelling Degree

The swelling index is a crucial parameter because it is closely related to the integrity of food packaging materials in water media. The swelling progress of chitosan film is due to hydrophilic amino groups which interact with water molecules via hydrogen bonding. Then, the diffusion of water into polymeric matrix resulting making polymer chain relaxed and hydrated therefore leading to expansion in whole polymer network before degradation [49,50]. The degree of swelling of control chitosan film after 24 h immersed in water was recorded at 50% (Table 2). All PBLO incorporated chitosan films show higher swelling degree compared to control chitosan. This result is coincided with XRD analyses as the addition of PBLO may convert semi-crystalline in chitosan film toward more amorphous structure. Therefore, the swelling index increased progressively as the incorporated PBLO content augmented. This is opposite with chitosan film incorporated with *Piper betle* Linn leaf extract in our previous report [23]. The uptrend was likely due to the formation of chemical interaction between functional groups ($-\text{OH}$, $-\text{NH}_2$) of chitosan and high molecular phenolic groups in PBLO that exerts the partial disruption in polymeric network during 24 h swelling test [17].

Table 2. The swelling degree and mechanical properties of pCS, pCS-0.4PBLO, pCS-1PBLO, and pCS-1.2PBLO.

Sample Codes	Tensile Strength (MPa)	Elongation at Break (%)	Elastic Modulus (MPa)	Swelling Degree (%)
pCS	15.26 $\pm$ 0.38	17.49 $\pm$ 1.09	111.96 $\pm$ 13.31	50.10 $\pm$ 2.56
pCS-0.4PBLO	11.98 $\pm$ 0.41	24.97 $\pm$ 0.88	51.00 $\pm$ 1.48	52.57 $\pm$ 3.01
pCS-1PBLO	7.71 $\pm$ 0.36	20.62 $\pm$ 0.88	32.16 $\pm$ 2.85	62.05 $\pm$ 4.50
pCS-1.2PBLO	5.42 $\pm$ 0.40	17.06 $\pm$ 1.06	25.95 $\pm$ 1.58	70.54 $\pm$ 2.66

3.9. Antibacterial Activity

The antibacterial activities of films were tested by using agar diffusion and liquid incubation methods [25,26]. The bacterial viability levels were evaluated using the inhibition diameters of ≥ 28 mm, 16–28 mm, 12–16 mm, and ≤ 12 mm for the strong, moderate, mild, and no activities, respectively [51]. As shown in Figure 6, the pCS-PBLO films were evaluated as moderate activities against the *E. coli*, *P. aeruginosa*, and *S. typhi*, except the *S. aureus*, in which the *S. typhi* was most vulnerable. In addition, the inhibition activity significantly increased in accordance with the increase of the PBLO from 0.4% to 1% (*v/v*). However, the PBLO at concentration of 1.2% (*v/v*) did not reveal further increased inhibition efficiency. In contrast, no inhibition zone was observed with the control pCS (Figure S1). This result is accordance with previous reports that the antibacterial activity of chitosan in solid form was negligible because only bacteria directly exposing with active sites were inhibited [16,42]. Similar finding showed that the eugenol, mainly consisted in PBLO, may disrupt the cytoplasmic membrane of *S. typhi* and rise the permeability in bacterial cell [52]. The cell death was resulted from the ion leakage and loss of intracellular proteins. The compactness in polymeric network of pCS-PBLO film may hinder the exposure between bacterial cells and phenolic molecular [16,53].

Figure 6. The antibacterial activity of pCS, pCS-0.4PBLO, pCS-1PBLO, and pCS-1.2PBLO against *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhimurium*.

Liquid inoculation tests showed that inhibition activities of the pCS-PBLO to *E. coli* strain was observed at all tested-PBLO concentrations of 0.4%, 1%, and 1.2% (Table 3, Figure S2). However, at low PBLO concentration of 0.4%, the number of bacteria increased after 8 h of co-inoculation. Interestingly, at PBLO concentration of 1.2%, bacteria were killed after 24 h of co-inoculation. It found that the antibacterial activity of CS-PBLO film was lower than film containing *Piper betle* Linn extract [23], which was due to the composition of volatile phenolic compound during oil preparation process. In general, the addition of PBLO significantly improved the antibacterial activity of control chitosan film. These results indicated that the PBLO can be used as an antibacterial agent in producing the effective food packaging film products.

Table 3. Results of time-dependent assays for *E. coli*.

Sample Codes	Total Colony Forming Units (CFU/mL)				
	0 h	4 h	8 h	11 h	24 h
pCS	7.50×10^9	8.30×10^9	GO	GO	GO
pCS-0.4PBLO	7.80×10^9	7.40×10^9	5.70×10^9	6.20×10^9	12.30×10^9
pCS-1PBLO	7.60×10^9	5.45×10^9	4.65×10^9	2.35×10^9	2.00×10^8
pCS-1.2PBLO	7.50×10^9	4.65×10^9	1.45×10^9	4.00×10^8	++++

(++++): strong inhibition; GO: growth over.

3.10. Antioxidant Activity

In addition to bactericidal activity, PBLO as an antioxidant source incorporated with chitosan film may scavenge the free radicals preventing the oxidation of nutritional components and food deterioration. The antioxidant activity of pCS-PBLO films was evaluated through the half maximal inhibitory concentration (IC_{50}) which was determined as the concentration of sample required to scavenge 50% of DPPH radicals. The lower the IC_{50} value is, the stronger the antioxidant activity is. Herein, IC_{50} values of films containing 0.4, 1.0, and 1.2% (*v/v*) PBLO were resulted in 48.43, 44.86, and 42.49 $\mu\text{g/mL}$, indicating that antioxidant activity of resultant films increased with increasing incorporated PBLO concentration. The phenolic compound bearing $-\text{OH}$ groups in PBLO may contribute to antioxidant mechanism as they scavenge $\text{DPPH}\cdot$ by donating either electron or hydrogen

radical to free radical [54]. The IC_{50} index was not documented for control chitosan film. Although active amino groups presenting in chitosan structure can react with free radical, the inhibitory efficiency of neat chitosan to DPPH was lower than 50% [55]. It can be observed the antioxidant activity of chitosan film was improved by the incorporation of PBLO; however, this property was lower as compared to that in the film containing *Piper betle* Linn extract [23]. These results revealed that PBLO containing chitosan films hold great antibacterial and antioxidant activities making them ideally suitable for food packaging film.

3.11. Preservative Application

The king orange, a variety of citrus fruit from Vietnam, was chosen for the test because it abundantly contained high nutritional values, especially rich in vitamin C. The appearance of king oranges with and without chitosan-based coatings, stored at 25 °C for 2 weeks were showed (Figure 7). The uncoated samples (control) were utilized to compare with coated king oranges. The bright green rind is observed for control sample while coated oranges reveals shinier and bright green rind surface on the first day. However, the rind of control sample moved to light yellow in color after storage 5 days but those do not occur for coated oranges. After 10 days, the bright yellow color is clearly viewed in control and chitosan coating on the orange surface begins to peel off. Interestingly, pCS-0.4PBLO coated orange still keep bright green and shiny skin after 10 days and up to two weeks. This is supported by the fact that higher work of adhesion and spreading coefficient of pCS-0.4PBLO solution over orange skin as compared to chitosan based coating liquids (Table S2). For cross-section images, the dense and compact texture are obviously observed for the king orange coated by chitosan and chitosan-0.4PBLO solution.

Figure 7. The visual appearance of king oranges with and without coating stored at 25 °C for two weeks.

4. Conclusions

The present work reports the successful incorporation of *Piper betle* Linn oil in chitosan film (pCS-PBLO film) to produce potential active food packaging material. The microstructure of the pCS-PBLO film exhibited discontinuous and heterogeneous appearance by the presence of oil droplets on the film surface. Furthermore, the inter-molecular interaction among functional groups in chitosan and *Piper betle* Linn oil was confirmed by FTIR, XRD, and TGA analyses. The mechanical behavior and swelling degree were directly affected by the incorporation of PBLO. The pCS-PBLO film revealed the antibacterial and antioxidant activity based on phenolic compounds such as acetyleugenol, eugenol, 4-allyl-1,2-diacetoxybenzen, chavicol acetate in PBLO components; however, the bioactivity of resultant films was lower as compared to that in chitosan film containing *Piper betle* Linn extract in our previous report. Moreover, the great UV-barrier property and strong antioxidant activity was the light of the pCS-PBLO film. In particularly, the pCS-PBLO film may protect the shelf life of king oranges from the food deterioration for up to two weeks, suggesting that the film can be proposed for effective food packaging application.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2079-6412/11/3/351/s1>, Figure S1: Inhibitory effects of PBLLE incorporated chitosan films against the growth of *Staphylococcus aureus*, *Salmonella Typhimurium*, *Escherichia coli*, and *Pseudomonas aeruginosa* were observed via real photos taken after 24 h exposure, Figure S2: Inhibitory effects of PBLLE incorporated chitosan films against the growth of *Escherichia coli* was observed via real photos taken after 0 h, 4 h, 8 h, 11 h and 24 h exposure Table S1: Degradative temperature and mass loss of pCS, pCS-0.4PBLO, pCS-1PBLO, and pCS-1.2PBLO, Table S2: Surface tension, work of adhesion, and spreading coefficient of chitosan solution with and without PBLO.

Author Contributions: T.T.N.: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Writing-original draft; T.-T.T.N.: Formal analysis; T.V.T.: Methodology; L.V.T.: Data curation, Methodology; L.T.D.: Data curation, Methodology; V.T.T.: Writing-original draft, Writing-review & editing. All authors have read and agreed to the published version of the manuscript.

Funding: The study was supported by The Youth Incubator for Science and Technology Program, managed by Youth Development Science and Technology Center-Ho Chi Minh Communist Youth Union and Department of Science and Technology of Ho Chi Minh City, the contract number is “26/2019/HĐ-KHCN-VU”.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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